NeuroToxicology 31 (2010) 277–290

Contents lists available at ScienceDirect

NeuroToxicology

Quantitative assessment of neurite outgrowth in human embryonic stem cell-derived hN2^{TM} cells using automated high-content image analysis☆

Joshua A. Harrill [a], Theresa M. Freudenrich [a], Dave W. Machacek [b], Steven L. Stice [b,c], William R. Mundy [a,*]

[a] Systems Biology Branch, Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, NC 27711, United States
[b] ArunA Biomedical, Athens, GA 30602, United States
[c] Regenerative Bioscience Center, University of Georgia, Athens, GA 30602, United States

ARTICLE INFO

Article history:
Received 14 December 2009
Accepted 17 February 2010
Available online 25 February 2010

Keywords:
Neurite outgrowth
High-content analysis
Human embryonic stem cell-derived neural culture

ABSTRACT

Throughout development neurons undergo a number of morphological changes including neurite outgrowth from the cell body. Exposure to neurotoxic chemicals that interfere with this process may result in permanent deficits in nervous system function. Traditionally, rodent primary neural cultures and immortalized human and non-human clonal cell lines have been used to investigate the molecular mechanisms controlling neurite outgrowth and examine chemical effects on this process. The present study characterizes the molecular phenotype of hN2^{TM} human embryonic stem cell (hESC)-derived neural cells and uses automated high-content image analysis to measure neurite outgrowth *in vitro*. At 24 h post-plating hN2^{TM} cells express a number of protein markers indicative of a neuronal phenotype, including: nestin, β_{III}-tubulin, microtubule-associated protein 2 (MAP2) and phosphorylated neurofilaments. Neurite outgrowth in hN2^{TM} cells proceeded rapidly, with a majority of cells extending one to three neurites by 48 h in culture. In addition, concentration-dependent decreases in neurite outgrowth and ATP-content were observed following treatment of hN2^{TM} cells with either bisindolylmaleimide I, U0126, lithium chloride, sodium orthovanadate and brefeldin A, all of which have previously been shown to inhibit neurite outgrowth in primary rodent neural cultures. Overall, the molecular phenotype, rate of neurite outgrowth and sensitivity of hN2^{TM} cells to neurite outgrowth inhibitors were comparable to other *in vitro* models previously characterized in the literature. hN2^{TM} cells provide a model in which to investigate chemical effects on neurite outgrowth in a non-transformed human-derived cells and provide an alternative to the use of primary rodent neural cultures or immortalized clonal cell lines.

Published by Elsevier Inc.

1. Introduction

During the differentiation of precursor cells to a committed neuronal lineage, newly formed neurons undergo a series of extensive morphological changes as they mature including emergence of neurites, neurite outgrowth, neurite branching and establishment of cell–cell contacts (i.e. synaptogenesis). These morphological changes are necessary, although not sufficient, for the formation of the intricate network of neural circuits that facilitate nervous system function (Sanes et al., 2006). Early pre- and post-natal exposure to neurotoxic compounds can interfere with these developmental events and could potentially result in deficits in nervous system function in later life stages (Rice and Barone, 2000; Costa et al., 2004; Grandjean and Landrigan, 2006). Neurite outgrowth, a critical component of this developmental chain of events, can be recapitulated *in vitro* using a variety of cell models, such as nervous system derived clonal cell lines and primary neural cultures from the mammalian CNS. These models have become valuable tools for studying the molecular mechanisms that control neurite outgrowth (Zhang et al., 2009a,b; Yu and Malenka, 2003; Redmond et al., 2002; Jin et al., 2003; Khaibullina et al., 2004) and for investigating the mechanism(s)-of-action for known developmental neurotoxicants (Yamauchi et al., 2007; Lein et al., 2000; Howard et al., 2005; Audesirk et al., 1991). It has also been proposed that *in vitro* measures of neurite outgrowth can be useful in high-throughput screening assays (Radio et al., 2008, 2010) as a means to identify potential developmental neurotoxicants (Radio and Mundy, 2008).

☆ This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

* Corresponding author at: USEPA, Integrated Systems Toxicology Division, B105-06, Research Triangle Park, NC 27711, USA. Tel.: +1 919 541 7726.
E-mail address: mundy.william@epa.gov (W.R. Mundy).

0161-813X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.neuro.2010.02.003

A number of recent publications advocate the use of *in vitro* cell culture models as tools for efficient identification and prioritization of chemicals that may be hazardous to humans (NRC, 2007; Coecke et al., 2007; Lein et al., 2005). Specifically, a report by the National Academy of Sciences entitled 'Toxicity testing in the 21st century: a vision and a strategy' emphasizes the use of *in vitro* models derived from human tissues (NRC, 2007). The impetus for this point-of-view being that use of *in vitro* toxicity assays in human-derived cells, as opposed to cells from non-human mammalian species, may decrease some of the uncertainties involved in evaluating the effects of chemicals and applying that knowledge to address human risk (i.e. interspecies extrapolation) (NRC, 2007). In the case of assessing neurite outgrowth, there are a number of immortalized and tumor-derived neural cell lines of human origin currently available (Radio and Mundy, 2008; Harry and Tiffany-Castiglioni, 2005) as well as reliable methods for the culture of primary rodent neurons (Higgins and Banker, 1998). However, transformed clonal cell lines or rodent primary neural cultures may not accurately represent human nervous system biology (LePage et al., 2005; Allen et al., 2005). The response or sensitivity of neural cultures to toxic compounds may differ across species or across models as noted in previous reports examining the effects of ethanol, staurosporine and mercury on the processes of neural development *in vitro* (Breier et al., 2009; Moors et al., 2009; Cedrola et al., 2003). In the context of evaluating chemicals as potential developmental neurotoxicants in humans, a human stem cell-derived culture model may prove more informative than transformed cell lines and also circumvent problems associated with primary human neural cell availability (McNeish, 2004).

Recent advances in stem cell biology have resulted in methods in which embryonic stem cells of human origin can be differentiated along a neuronal lineage and grown in dissociated cultures (Reubinoff et al., 2001; Zhang et al., 2001). Given the proper extracellular cues and growth substrate, the maturing cells can display morphological characteristics and express a number of protein markers indicative of a neuronal lineage (Reubinoff et al., 2001; Zhang et al., 2001; Shin et al., 2006). These stem cell-derived models may serve as valuable tools for examining chemical effects on neuronal maturation, including neurite outgrowth, using human cells. In the context of high- to medium-throughput chemical screening, the use of stem cell-derived neuronal cultures also has some potential caveats. Namely, the time- and labor-intensive process of differentiating a proliferative population of stem cells (hESC) to a population of terminally differentiated neurons, which can take weeks (Reubinoff et al., 2001; Zhang et al., 2001; Shin et al., 2005). The present study describes the phenotypic characteristics and measures neurite outgrowth in hN2^{TM} cells, a novel, commercially available, hESC-derived neuronal model which is provided in a pre-differentiated state for rapid end user applications (ArunA Biomedical, Athens, GA).

The hN2^{TM} cell line is derived from neuroepithelial cells of WA09 hESC (Thomson et al., 1998) origin according to a previously described protocol (Shin et al., 2005, 2006). Importantly, as opposed to other methods of deriving neural progenitors through three-dimensional neurosphere and embryoid body formations (Reubinoff et al., 2001; Zhang et al., 2001), these adherent monolayer cultures are uniformly exposed to growth factors and/or morphogens throughout their propagation. Prior to differentiation into hN2^{TM} cells the population was confirmed karyotypically normal, >95% nestin positive and <3% OCT-4 positive (Shin et al., 2006). The cells were produced in bulk by propagation for an additional 2 weeks beyond the neuroepithelial stage by removal of bFGF from the media and cryopreserved (ArunA Biomedical, Athens, GA) for end user applications. In the present study, the utility of dissociated hN2^{TM} cultures as an *in vitro* model for neurite outgrowth was assessed using automated high-content image analysis (HCA). In addition, the molecular phenotype of these cells was examined using immunocytochemical staining.

2. Methods

2.1. Materials

hN2^{TM} human neural cells, growth media and supplements were obtained from ArunA Biomedical, Inc. (Athens, GA). The growth substrates poly-L-lysine and laminin were purchased from Sigma–Aldrich (St. Louis, MO). Bisindolylmaleimide I (Bis1) and brefeldin A were purchased from Calbiochem, Inc. (San Diego, CA). Dimethyl sulfoxide (DMSO, dosing vehicle), lithium chloride (LiCl) and sodium orthovanadate (Na_3VO_4) were purchased from Sigma–Aldrich (St. Louis, MO). U0126 was purchased from Promega Corp. (Madison, WI). Hoechst 33258 dye, immunocytochemical staining buffer (ISB), mouse monoclonal antibody against β_{III}-tubulin and DyLight® 488-conjugated rabbit anti-mouse IgG secondary antibody were components of a Cellomics® Neurite Outgrowth HitKit^{TM} purchased from ThermoFisher Scientific, Inc. (Waltham, MA). Mouse monoclonal antibodies for microtubule-associated protein 2 (MAP2) and nestin were purchased from Millipore, Inc. (Billerica, MA). Mouse monoclonal antibody SMI-312 which detects phosphorylated forms of a variety of axonal neurofilaments was purchased from Covance, Inc. (Princeton, NJ).

2.2. Cell culture

Costar® 96-well polystyrene cell culture dishes (Corning, Inc., Corning, NY) were coated with a solution of 50 μg/ml poly-L-lysine in sterile H_2O for 2 h (37 °C), rinsed once with sterile H_2O and then coated with a solution of 20 μg/ml laminin in sterile phosphate-buffered saline (PBS) for 2 h. Plates were then rinsed once with warm PBS prior to plating of hN2^{TM} cells. Cells were stored at −70 °C and thawed at time of use. After thawing at 37 °C, cells were suspended in serum-free ArunA basal medium supplemented with ArunA Neural Supplement (ANS^{TM}), leukemia inhibitory factor (LIF, 10 ng/ml), penicillin (50 U/ml), streptomycin (50 μg/ml) and 2 mM L-glutamine. A small aliquot of cells were then stained with 0.4% trypan blue and counted on a hemocytometer. Live cell yields post-thawing ranged from 60 to 80%. Cells were plated at densities ranging from 2500 to 10,000 cells/well (8.33×10^3 to 3.33×10^4 cells/cm^2, respectively) based on the number of live cells counted. The number of cells per cm^2 (i.e. plating density) was calculated by dividing the number of cells per well by the well area (0.3 cm^2). Cells were maintained in a humidified incubator at 37 °C with a 95% air/5% CO_2 atmosphere.

2.3. Chemical treatment

Concentration ranges for the five test compounds were as follows: brefeldin A (0.01, 0.03, 0.1, 0.3, 1 μM), Bis1 (0.1, 0.3, 1, 3, 10 μM), U0126 (0.3, 1, 3, 10, 30 μM), sodium orthovanadate (1, 3, 10, 30, 100 μM) and lithium chloride (0.3, 1, 3, 10, 30 mM). Guidance for concentration range selection was based on previously published works cited in Table 1. Stock solutions (1000×) of the highest tested concentration of Bis1, brefeldin A and U0126 were prepared in pure DMSO and stock solutions for the remainder of the concentration ranges were prepared by serial dilution in DMSO. Dosing solutions for each chemical concentration were prepared by diluting stock solutions 1:100 in ArunA basal media. Stock and dosing solutions of LiCl and Na_3VO_4 were prepared using the same method, save that stock solutions were prepared in ArunA basal medium as opposed to DMSO. 10 μl of dosing solutions were then added to the cell culture wells